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| (54) Title: DIAGNOSTIC TESTS MEASURING GELATINASE/INHIBITOR COMPLEXES FOR DETECTION OF AG- GRESSIVE AND METASTATIC CANCER | | |
| (57) Abstract Diagnostic agents and methods for detecting the presence of metastatic activity in biological samples such as plasma are disclosed. The agent and method preferably immunologically detect matrix metalloproteinases in complexed form with endoge- nous inhibitors of MMP's. A kit for detecting the metalloproteinases is also disclosed. | | |

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DIAGNOSTIC TESTS MEASURING GELATINASE/INHIBITOR COMPLEXES FOR DETECTION OF AGGRESSIVE AND METASTATIC CANCER

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BACKGROUND OF THE INVENTION

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The present invention relates to improvements in the diagnosis of metastatic disease. In particular, the invention relates to techniques for detecting the presence of proteolytic enzymes associated with metastatic disease.

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Although a cure for most forms of cancer remains elusive, early detection and treatment have historically provided the best prognosis. Considerable effort over the years has been directed to developing diagnostic tests which give an indication of the presence of metastatic disease at an early stage.

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Recently, a relationship between matrix metalloproteinases (MMP's) and metastasis has been suggested. MMP's are a family of closely related metal-dependent endopeptidases secreted by mesenchymal cells. For example, gelatinase A (MMP-2 or 72 kDa gelatinase/type IV collagenase) and gelatinase B (MMP-9 or 92 kDa gelatinase/type IV collagenase) have been identified as playing a major role in cancer invasion and metastasis. Stromelysin and PUMP (Putative Metalloproteinase), metalloproteinases with broader substrate specificity, are also thought to participate in the metastatic process. Three of these metalloproteinases have been identified in mammalian plasma.

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Metastasis is a complex sequence of events in which malignant cells detach and disseminate from a primary tumor. The malignant cells invade adjacent tissue, penetrate into blood and lymphatic vessels, circulate to distant sites and eventually attach to and penetrate tissues in distant organs, thereby proliferating the malignancy. An important part of the metastatic process is the degradation of extracellular basement membranes by various proteolytic enzymes.

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1 Basement membranes are collagen-containing connective
tissues which form a tough continuous sheet and separate the
various cell layers such as the epithelial, endothelial and
parenchymal cells from interstitial connective tissue.

5

Proteolytic enzymes associated with the metastatic
process are found circulating in the form of activated
enzyme (free enzyme), latent free enzyme and complexed with
endogenous proteinase inhibitors. Only the active forms,
10 however, digest the connective tissue substrates. The
body's own natural defense mechanisms rapidly inactivate
MMP's by complexing the enzymes with specific tissue
inhibitors of metalloproteinases (TIMP's). Such inhibitors
found in the tissues and circulating in the plasma include
15 TIMP-1, TIMP-2 and also alpha-2 macroglobulin which is
primarily in blood.

In spite of the relationship between elevated
levels of certain destructive proteases and the presence of
20 metastatic disease, it has been difficult to use the
relationship to provide an accurate indication of metastatic
disease. In the past, it has only been possible to detect
free or activated forms of the metalloproteinases, leaving a
substantial portion of the inactivated enzymes complexed
25 with inhibitors undetected. Recently it has been shown,
however, that latent gelatinase A forms complexes with TIMP-
2 and latent gelatinase B forms complexes with TIMP-1.
Thus, these gelatinases can exist outside the cell in
complexed forms with TIMPs.

30

U.S. Patent No. 4,677,058 discloses purifying
and detecting type IV collagenase antigens from malignant
tumor cells. Similarly, U.S. Patent No. 4,808,528 discloses
antibodies specific to type IV collagenase enzyme antigens.
35 U.S. Patent No. 4,816,400, a division of the '058 patent,
supra, discloses immunological determination of type IV

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1 collagenase antigens using polyclonal and monoclonal
antibodies. None of these references, however, disclose
detecting collagenase enzyme-inhibitor complexes associated
with metastatic disease.

5

In spite of the investigation of the role of
metalloproteinases in metastatic disease, the total amount
of MMP's being released and thus the actual metastatic
activity has not been detectable. Indeed, due to the
10 usually rapid inactivation of activated MMP's by TIMP's,
measuring only free, circulating MMP's would fail to
indicate the presence of many underlying diseases.
Determining whether or not the complexes formed between
MMP's and TIMP's form a more reliable and a sensitive
15 diagnostic tests has yet to be investigated.

It is therefore an object of the present invention to
provide highly specific and reliable diagnostic agents and
methods to determine the presence of metastatic disease
20 based on measuring matrix metalloproteinase inhibitor
complex levels.

Other and further objects and advantages of the
present invention will become apparent to those skilled in
25 the art from a consideration of the following description
taken together with the accompanying figure.

SUMMARY OF THE INVENTION

30 The present invention includes diagnostic agents and
methods useful in the detection of metastatic activity in
biological samples such as human plasma. The invention
includes a first substance capable of immunologically
reacting with enzymes broadly described as matrix metallo-
35 prot inases (MMP's). Such MMP's include gelatinase A 72 kDa
type IV collagenase/gelatinase (MMP-2), gelatinase B 92 kDa

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- 1 type IV collagenase/gelatinase (MMP-9), stromelysin (MMP-3),
putative metalloproteinase (PUMP), partial breakdown
products of these proteins and combinations thereof. The
invention further includes a second substance which is
5 immunologically reactive with endogenous inhibitors of the
tissue-degrading enzyme metalloproteinases or TIMP's.

The first immunologically-reactive substance is
preferably a monoclonal antibody having specificity for one
10 or more MMP enzymes or circulating breakdown products of
MMPs. Examples of such antibodies include monoclonal murine
anti-MMP-2, monoclonal murine anti-MMP-9 and monoclonal
murine anti-MMP-3, such that set forth by Bergmann, et al.
J. Clin. Chem. Clin. Biochem 27, 351-359 (1989) or Cell Tech
15 Lmt. (Slough, England). Alternatively, antibodies such as
rabbit polyclonal antibodies to native MMP's or peptide
components such as peptide sequences of the native MMP's may
be used.

20 The second immunologically responsive substance is
preferably an antibody having specificity for endogenous
inhibitors of MMP's, TIMP's or other associated proteins
which bind to MMP's in plasma or tissue. For example,
polyclonal rabbit anti-TIMP-1 or polyclonal anti-TIMP-2 as
25 described by Carmichael, et al. Proc. Natl. Acad. Sci. USA,
83:2407-2411 (1986) or the N.I.H. (Bethesda, MD) are
examples of such substances. Alternatively, monoclonal
antibodies to TIMP's may be selected.

30 The present invention also includes a method of
detecting metastatic activity in a biological sample and
a diagnostic kit. The method includes contacting a
diagnostic agent, such as that set forth above, with the
biologic sample and measuring the total amount of matrix
35 metalloproteinases present and TIMP complexes uncovered to
determine whether metastatic disease is present.

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1 The diagnostic agent, method and kit of the present
invention can be included as part of various immunoassay
techniques, particularly ELISA and most preferably sandwich-
type ELISA assays. Alternative immunoassay techniques such
5 as immunoblotting, immunofluorescent, radio-immunoassay,
fluorescence detection and/or enzyme assay methods are also
contemplated.

As a result of the present invention, significantly
10 more accurate determinations of metastatic activity are
obtained by detecting not only free metalloproteinases
and breakdown products of MMP's, but also complexes of
the enzymes formed with inhibitors. This is a dramatic
improvement over methods which only detect free or activated
15 MMP's and completely missed those enzymes complexed to the
inhibitor molecules. In addition, the assays described
herein provide independent verification of disease. Thus,
the results provided can supplement other tests and provide
additional data not obtained with other tests.

20

For a better understanding of the present invention,
reference is made to the following description, taken
together with the accompanying figure, and its scope will be
pointed out in the appended claims.

25

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 graphically demonstrates the results of
immunological assays carried out using sandwich-type ELISA
30 techniques to detect metastatic activity in patients using
the present invention.

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DETAILED DESCRIPTION OF THE INVENTION

The diagnostic agent and method of the present invention are based on the premise that high levels of certain metalloproteinases complexed to TIMP's in clinical samples strongly correlate with an underlying metastatic disease. Thus, while not necessarily indicating a particular type of cancer, the diagnostic agent and method of the present invention provide a means of indicating or affirming the presence of underlying metastatic disease in the biologic fluids of suspected patients.

Blood and/or plasma are the most common biologic fluids assayed for diagnostic tests. For purposes of the present invention, the term biologic fluids shall also include but not be limited to plasma, serum, tissue samples, aspirates, urine and tissue fluids. It is contemplated that the inventive diagnostic agent and method provide an indication of metastatic activity from any sample containing metalloproteinases in both free and complexed form, even in amounts as low as the nanogram per milliliter level.

The identification of metalloproteinases in clinical samples is preferably carried out using immunologic techniques. The immunologic techniques center around the use of specific antibody-antigen reactions which indicate a response to only specific antigens, in particular, those enzymes associated with metastatic disease. Within this genre of tissue-degrading enzymes are gelatinase A 72 kDa type IV collagenase/gelatinase (MMP-2), gelatinase B 92 kDa IV collagenase/gelatinase (MMP-9), stromelysin (MMP-3), putative metalloproteinase (PUMP's), and breakdown products of the proteinases and combinations thereof. For diagnostic purposes, it is of importance to note that the recognition of these antigenic substances is in both the free and complexed form.

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1 In order to achieve this result, the invention
includes a first substance having immunospecificity to free
metalloproteinases and complexed metalloproteinases. In a
preferred embodiment, monoclonal antibodies are prepared to
5 have the desired immunospecificity. For example, monoclonal
antibodies may be obtained from hybridomas obtained from
mice immunized by injection of 72 kDa procollagenase/type IV
procollagenase purified from human fibroblasts. See
Birkedal-Hansen, et al. Biochemistry 27, 6751-6758 (1988).
10 It is essential that the antibody bind to the specific
metalloproteinases when it is complexed to TIMP. Many
antibodies currently used can recognize free metallo-
proteinase, but they fail to bind to MMP in complexes with
inhibitors as described in more detail below. A non-
15 limiting list of suitable antibodies include murine
monoclonal anti-human 72 kDa or 92 kDa type IV collagenase/
gelatinase antibodies, or murine monoclonal antibodies to
stromelysins. One method of producing these antibodies
which recognize MMP's in complexes is to use MMP-TIMP
20 complexes as the immunogen in mice or rabbits.

The inventive diagnostic agent also includes a second
substance having particular specificity for the naturally-
occurring tissue inhibitors of metallo-proteinases (TIMP's),
25 including TIMP-1, TIMP-2, and alpha macroglobulin. These
antibodies need to be reactive to TIMP's in complexes with
MMP's.

Free TIMP-1 has a molecular weight of 28 kDa,
30 but in complexes with MMP's, it is identified as
approximately 95 kDa. Free TIMP-2 has a molecular weight of
22 kDa, but complexed with MMP's, it is identified as
multiple components with molecular weights between 23 kDa to
150 kDa. Because gelatinase A and gelatinase B breakdown
35 over time following activation or enzyme digestion, it is
anticipated that lower molecular weight products of these

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- 1 MMPs may circulate in complexes with TIMP's. The molecular weights of TIMP's in plasma have been identified using immunoblotting techniques of non-reduced SDS-PAGE polyacrylamide gels.

5

- Preferably, antibodies which achieve the necessary binding to complexed TIMP are prepared specific to MMP-TIMP complexes found in the biologic sample. For example, polyclonal antibodies to human procollagenase/pro-type IV collagenase can be produced in rabbits by laboratory procedures known in the art. A good source for the immunogens is to isolate MMP-TIMP complexes from human plasma using gelatin Sepharose chromatography to bind these antigens and dimethyl sulfoxide to elute the complexed proteins from the solid phase. These antigens are mixed with Freund's Adjuvant to enhance antibody response and are injected subcutaneously in rabbits on 3-5 occasions over a period of 4-6 weeks. Further examples of such polyclonal antibodies include rabbit anti-TIMP-1, rabbit anti-TIMP-2 and anti-alpha-2-macroglobulin. Suitable antibodies are also available from commercial laboratories such as Cell Tech Ltd. (Slough, England).

- In an alternative embodiment, monoclonal antibodies are prepared with specificity to the TIMP complexes such as MAC-015 from Cell Tech Ltd. Combinations of antibodies are also contemplated. The monoclonal antibodies can also be prepared using laboratory techniques known to those of ordinary skill in the art, such as that provided by Cooksley, et al. MATRIX 10:285-291, 1990, or from commercial laboratory sources such as Cell Tech Ltd. The antibodies employed in this assay could also react to a new immunogen consisting of peptide components derived from a portion of the TIMP molecule and a portion of the MMP molecule.
- 35 Likewise, neoantigens could be produced as a result of the complexing of TIMP and MMP's, thus resulting in a unique

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- 1 antigen that would be diagnostic of the formation of
complexes.

5 A preferred immunologic means of detecting
metalloproteinases is the Enzyme-Linked Immunosorbent Assay
(ELISA) method, and in particular the sandwich-type ELISA
format. This assay method includes introducing a biologic
sample between a capture layer of antibodies and a detection
layer of antibodies.

10

In this regard, diagnostic well plates such as
Immulon II 96 well microtiter plates available from
Dynatech, Alexandria, VA are first coated with a capture
antibodies directed to MMP's such as rabbit polyclonal
15 antibodies to human 72 kDa gelatinase or 92 kDa
progelatinase/type IV procollagenase. The capture antibody
is introduced into the wells in amounts ranging from about
10 μ l to about 200 μ l, with amounts of about 100 μ l being
preferred. The capture antibody is preferably diluted in a
20 suitable buffer such as 0.1 M NaHCO₃, pH 9.0 to about 1:200
concentration prior to introduction into the wells.

Binding of the capture antibody to the well is carried
out over a period of from about 4 to about 24 hours and
25 preferably about 18 hours after inoculation at temperatures
ranging from about 0 to about 10°C and preferably about 4°C.
Unbound antibody is thereafter removed by vigorously
inverting the plates. The wells containing the bound
antibody are next bathed with a bovine serum albumin/
30 bicarbonate buffer to block excess binding sites on the
wells. The bottom layer of the "sandwich" or capture layer
is completed by washing the plate thoroughly with a buffer
solution containing sodium phosphate, sodium chloride and
Tween 20.

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1 The ELISA technique further includes introducing
a biologic sample such as human plasma onto the capture
antibody layer. The samples are preferably prepared by
being diluted in an incubation-suitable buffer to about
5 a 1:10 concentration. One such solution contains 50 mM
sodium phosphate, 0.1 M sodium chloride, 0.02% Tween 20 and
0.1% bovine serum albumin (BSA). The samples are placed in
the well, incubated at a temperature ranging from about 25°C
to about 37°C, and preferably at about 37°C for a time
10 period of from about 1 hour to about 4 hours and preferably
about one hour.

The final part of the ELISA "sandwich" is a detection
layer containing an antibody specific to free TIMP-1 or
15 TIMP-2 recognizing TIMP in complexes. The wells containing
the capture antibody and sample are washed thoroughly before
introducing a detection antibody into the well such as
monoclonal murine anti-TIMP-1, anti-TIMP-2 antibodies or the
like. The antibodies are preferably added after being
20 diluted in an incubation buffer to about a concentration of
about 1:4000.

The wells are incubated for about 1 hour and
thereafter washed thoroughly and prepared using standard
25 ELISA techniques known to the art, such as including the
amplifying goat antibodies to mouse IgG and then alkaline-
phosphatase conjugated to streptavidin. The wells are then
washed and 100 microliters of substrate p-nitrophenyl
phosphate in buffer is added to generate a color reaction
30 which is read at A_{405} . The results of the assay are obtained
using any suitable reading device such as that available
from BioTek of Winooski, VT. In addition, the optimal
concentration of capture antibodies such as polyclonal
rabbit anti-gelatinase A, and detecting antibodies such as
35 monoclonal murine anti-TIMP-1, is determined using
checkerboard titration. In this procedure,

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- 1 the concentrations of different antibodies and antigens are
varied by serial dilution of the reagents to determine the
concentration of each reagent, giving the highest percentage
of true positive results and the lowest percentage of false
5 positive results.

While the present invention is not solely limited
to ELISA immunologic techniques, the ELISA technique is
particularly preferred since the method allows the artisan
10 to detect concentrations of a particular substance, in this
case MMP's, in nanogram per milliliter concentra-tions.
Other suitable immunological techniques include radio-
immunoassay (RIA), Western dot blot, dip stick, zymography
as well as immunologic techniques known to those of ordinary
15 skill in the art. For example, in certain techniques an
absolute-type reading, i.e., a color change, indicates the
presence of disease. In a preferred embodiment, a
quantitative or numeric value indicating the level of
enzyme-complex concentration or activity is provided thereby
20 allowing comparison to a reference standard.

In another aspect of the present invention, a kit
is provided for detecting the presence of metastatic disease
in a biologic sample. The kit provides the inventive
25 diagnostic agent as described herein and means for measuring
the total amount of MMP complexed with circulating
inhibitors thereof. In particular, the kit includes
antibodies, preferably monoclonal or polyclonal antibodies
against MMP's and a second type of antibodies, preferably
30 monoclonal or polyclonal antibodies directed against TIMP's.
The means for measuring can be any suitable means known to
those of ordinary skill in the art such as antibody coated
beads or other solid phase immobilized antibodies.
Measuring may also be accomplished by Enzyme Linked
35 Immunosorbent Assay (ELISA) radio-immunoassay, zymography
and the like techniques.

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1 Other immunologic detection means can be readily
adapted for use in connection with the diagnostic agent and
method of the present invention. It is intended that all
such alternative measuring and diagnostic means be included
5 within the scope of the present invention.

With particular regard to the antibodies included
herein, it will be appreciated by those of skill in the art
that such antibodies, both monoclonal and polyclonal types
10 are available from commercial sources such as Cell Tech Ltd.
of Slough, England, or can be prepared using standard
laboratory practices.

EXAMPLES

15 In the Examples set forth below, various aspects of
the present invention are set forth to provide further
appreciation of the invention. The Examples, however, are
not meant in any way to restrict the effective scope of the
20 invention.

EXAMPLE I

25 Sandwich-type ELISA Immunoassay
For Detection of MMP-2 and
MMP-2/TIMP-2 Complexes In Human Plasma

In this Example, a preferred immunoassay is prepared.
Initially, 96 well microtiter plates (Dynatech Immulon II,
Alexandria, VA) were coated with 100 μ l of polyclonal rabbit
30 anti-TIMP-2 (NIH, Bethesda, MD) diluted to a concentration
of 1:200 in 0.1 M NaHCO₃, pH 9.0 for 18 hours at 4°C. The
unbound anti-TIMP-2 was removed and 200 μ l of 1% bovine serum
albumin in bicarbonate buffer was added twice for 30 minutes
at 27°C to block excess binding sites on the wells. The
35 plate was washed 3 times with a washing buffer containing 50
mM sodium phosphate, pH 7.2, 0.1 M NaCl, 0.05% Tween 20.

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1 Twenty-eight human plasma samples and six 100 μ l
purified MMP-2/TIMP-2 complex standards were each diluted in
an incubation buffer containing 50 mM sodium phosphate, pH
7.2, 0.1M NaCl, 0.02% Tween 20, 0.1% bovine serum albumin,
5 (BSA), and separately added to individual wells for 2 hours
at 37°C. The wells were thereafter washed 3 times with
washing buffer. Monoclonal murine anti-MMP-2 obtained from
the University of Alabama, Birmingham, diluted 1:4000 in
incubation buffer, was added in an amount of 100 μ l per well.
10 The plates were incubated at 37°C for 1 hour.

The plates containing the ELISA sandwich were
completed by being washed 3 times and adding 100 μ l of biotin
labeled goat antibodies to mouse IgG, IgA, IgM (H&L chains)
15 in a concentration of 1:1000 before being incubated at 37°C
for 1 hour. The plates were again washed 3 times and
alkaline-phosphatase conjugated to streptavidin (1:1000
dilution, 100 μ l) was added to each well and the plates were
incubated for 30 min. at 37°C. The biotin and streptavidin
20 reagents are employed to amplify the signal from the
detecting antibody reagent, thus permitting the detection of
nonogram per ml concentrations of gelatinase-TIMP complexes.
Alkaline phosphatase conjugated to streptavidin is the
enzyme used to generate color from the p-nitrophenyl
25 phosphate substrate.

The wells were washed 3 times with PBS-Tween
phosphate-buffered saline with Tween detergent and 3 times
with water. 100 μ l of substrate p-nitrophenyl phosphate
30 (1mg/ml) in 0.1M glycine, 1mM MgCl₂, 1mM ZnCl₂, pH 10.4 was
added and after 30-90 minutes at room temperature, the
plates containing both the human plasma samples and the
purified standards were read at A₄₀₅ in a Microplate
Autoreader (BioTek EL 309. Winooski, Vt.). Quantification
35 of MMP-2/TIMP-2 complexes was made by extrapolation from a
log-log linear regression curve employing varying

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- 1 concentrations of purified MMP-2/TIMP-2 complex as standard.
The results are set forth in the Table below and are
graphically illustrated in Fig. 1.

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TABLE

| | <u>DIAGNOSIS</u> | <u>STAGE</u> | <u>MMP-2</u> <u>(ng/ml)</u> | <u>MMP-2/</u> <u>TIMP-2*</u> <u>(units/ml)</u> | <u>MMP-9</u> <u>(ng/ml)</u> |
|----|----------------------|--------------|---------------------------------|--|--------------------------------|
| 5 | | | Norm. Conc.= <u>0-828</u> | Norm. Conc.= <u>0-694</u> | Norm. Conc.= <u>0-31</u> |
| | <u>CONTROLS</u> | | | | |
| 10 | A | | 610 | 288 | 0 |
| | B | | 716 | 458 | 14 |
| | C | | 677 | 423 | 17 |
| | D | | 488 | 277 | 18 |
| | E | | 615 | 271 | 8 |
| | F | | 823 | 596 | 36 |
| | G | | 496 | 556 | 1 |
| | H | | 617 | 188 | 1 |
| | I | | 1381 | 604 | 25 |
| 15 | J | | 633 | 173 | 5 |
| | K | | 683 | 434 | 2 |
| | L | | 616 | 446 | 0 |
| | <u>GI CANCER</u> | | | | |
| 20 | A | 3 | 480 | 1429 | 63 |
| | B | 3 | 774 | 377 | 4 |
| | C | 4 | 591 | 657 | 5 |
| | D | 4 | 312 | 406 | 22 |
| | E | 4 | 664 | 933 | 126 |
| | F | 4 | 658 | 596 | 4 |
| | G | 4 | 908 | 3277 | 45 |
| | H | 2 | 500 | 779 | 1 |
| | <u>BREAST CANCER</u> | | | | |
| 25 | A | 4 | 622 | 804 | 1 |
| | B | 1 | 734 | 509 | 0 |
| | C | 3 | 1145 | 2428 | 51 |
| | D | 3 | 759 | 382 | 4 |
| | E | 4 | 786 | 2429 | 5 |
| | F | 4 | 1226 | 1278 | 71 |
| | G | 4 | 687 | 639 | 41 |
| | H | 3 | 734 | 593 | - |
| 30 | | | | | |

Key

35 *Units/ml based on a reference standard purified from human plasma using gelatin sepharose chromatography followed by gel filtration chromatography.

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- 1 As shown in the above Table, the normal range was based
on the mean \pm 2 S.D. of 12 normal plasma samples collected
in EDTA anticoagulant. Four out of eight GI cancer patient
specimens and four out of eight breast cancer patient
5 specimens (50%) had significantly increased levels of MMP-
2/TIMP-2 complexes. These patients tended to have advanced
cancer, indicating that this assay would be predictive of
patients with metastatic cancer.
- 10 The MMP-2/TIMP-2 complex assay provided results that were
independent from isolated measurements of either MMP-2 alone
or MMP-9 alone. Some patients had elevations of MMP-2/TIMP-
2 complexes and normal levels of MMP-2 or MMP-9, indicating
that multiple different assays will be supplemental in
15 enhancing the diagnostic utility of these assays. Results
of MMP-2 measurements alone have not proven to be useful in
the diagnosis of metastatic cancer. Furthermore, it is
critical that the blood specimens obtained from patients be
anticoagulated such as with EDTA since the levels of MMP's
20 are falsely elevated in serum as a result of release of
MMP's especially MMP-9 or gelatinase B from white blood
cells during the clotting process.

EXAMPLES II-IX

- 25 In these Examples, sandwich ELISA assays were prepared in
a manner similar that set forth in Example I, except that
the capture and detection antibodies were varied so that
different matrix metalloproteinases could be detected. The
30 various combinations of immunoassay detection systems
provide the clinician with a battery of diagnostic assays
useful in the identification, diagnosis, treatment and
determination of prognosis in patients with various types of
cancer. The illustrative assays are set forth below.

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| EXAMPLE | II | III | IV | V | VI | VII | VIII | IX |
|---|--------|--------|--------|--------|--------|--------|--------|--------|
| CAPTURE ANTIBODY TO DETECT TIMP SHOWN | TIMP-1 | TIMP-1 | TIMP-2 | TIMP-1 | TIMP-2 | TIMP-2 | TIMP-2 | TIMP-1 |
| DETECTION ANTIBODY TO DETECT MMP SHOWN | MMP-9 | MMP-3 | MMP-3 | MMP-1 | MMP-1 | MMP-2 | MMP-9 | MMP-2 |

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The above assays can be combined with other diagnostic methods such as (CEA) carcinoembryonic antigen measurements useful for colon cancer diagnosis and CA-125 (useful in ovarian cancer diagnosis) as a further or confirmational indicator of tumor growth and/or metastatic disease. It is to be understood that the above listed combinations are illustrative and in no way represent the complete range of possible assays which can be prepared in accordance with the present invention.

25

EXAMPLE X

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35

In this Example, a sandwich ELISA format immunoassay was prepared in a manner such as that set forth in Example I. The assay in this Example, however, contained mouse monoclonal antibodies to human TIMP-1 MAC-015 antibody from Cell Tech Lmt. to capture TIMP-1/MMP-9 complexes and biotinylated monoclonal mouse antibodies to 92 kDa matrix metalloproteinases prepared according to the method set forth

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1 by Bergmann, et al. supra, the disclosure of which is
incorporated by reference herein. The assay was used to
compare the levels of these complexed antigens present in the
5 plasma of patients with various types of cancer with those of
non-cancerous controls. The cancer group was further defined
as patients with gastrointestinal cancers (GI) and patients
with female genitourinary tract (GU) cancers such as cancers
of the ovary, cervix, vagina and uterus. The results are set
forth in the Table below.

10

TABLE

| | | | | | |
|----|------------------------|---|---------------------------------------|--|-------------------------------------|
| 15 | GROUP n=sample size | MMP-9/ TIMP-1 COMPLEX INCREASE ONLY DETECTED | MMP-9 INCREASE ONLY DETECTED | MMP-9/ TIMP-1 COMPLEX AND MMP-9 INCREASE DETECTED | TOTAL NUMBER OF POSITIVES (%) |
| 20 | Controls n=49 | 1 | 1 | 1 | 3 (6%) |
| | GI cancer n=94 | 14 | 14 | 7 | 35 (37%) |
| 25 | GU cancer n=23 | 8 | 3 | 4 | 15 (65%) |

Referring now to the Table, it can be clearly seen that there
are significant analytical advantages in measuring the levels
30 of MMP complexes in addition to free MMP's to detect or
confirm metastatic disease.

The above data dramatically illustrate this point for
patients with GU cancer. Consider that by measuring only
35 MMP-9, the clinician was able to confirm the presence of
metastatic disease in only 3 of 23 (13%) known metastatic

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1 plasma samples. However, when the clinician also assays
complexes of the enzyme and combinations of free enzymes and
complexes, the summation of all assays provides a much more
accurate diagnostic indicator. Moreover, the data obtained
5 from measuring TIMP/MMP complexes is independent from that
obtained by measuring MMP-9 assays alone. The present
invention, therefore, provides supplemental information
regarding metastatic disease. Patients with other types
of cancer may have elevations of different types of MMP
10 complexes thus necessitating the performance of a battery of
different tests such as that set forth in Examples II-IX to
optimize the diagnostic potential of detecting aggressive
cancer at an earlier stage.

15 The above Example provides further evidence of a strong
link between the invasive/metastatic process and the
increased levels of metalloproteinases, both free and with
inhibitor complexes in human plasma. Moreover, there is
also a positive correlation between highly metastatic cancer
20 cell lines (phenotypes) and increased secretion of certain
enzyme complexes. The practitioner, therefore, can custom
tailor the assay, for example, by substituting capture and
detection antibodies to measure specific antigens. It is
contemplated that assays for specific metastatic diseases
25 such as breast and/or gastrointestinal cancers could be
developed based on the results obtained from subjecting a
biologic sample to a battery of specific antibodies.

While there have been described what are presently
30 believed to be the preferred embodiments of the invention,
those skilled in the art will realize that changes and
modifications may be made thereto without the parting from
the spirit of the invention, and it is intended to claim
all such changes and modifications as fall within the true
35 scope of the invention.

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WHAT IS CLAIMED IS:

1. A diagnostic agent for detecting the presence of metastatic activity in a biologic sample, comprising:
 - a) a first immunologically responsive substance capable of reacting with matrix metalloproteinases; and
 - 5 b) a second substance capable of reacting with endogenous inhibitors of matrix metalloproteinases.
2. The diagnostic agent of Claim 1, wherein said matrix metalloproteinases are selected from the group consisting of 72 kDa type IV collagenase/gelatinase A (MMP-2), 92 kDa type IV collagenase/gelatinase B (MMP-9), stromelysin (MMP-3),
5 putative metalloproteinase (PUMP) and mixtures thereof.
3. The diagnostic agent of Claim 1, wherein said first responsive substance is capable of reacting with breakdown products of matrix metalloproteinases.
4. The diagnostic agent of Claim 1, wherein said first responsive substance is selected from the group consisting of monoclonal antibodies, polyclonal antibodies and mixtures thereof.
5. The diagnostic agent of Claim 4, wherein said first responsive substance is selected from the group consisting of murine monoclonal anti-human 72 kDa type IV gelatinase antibodies, 92 kDa type IV gelatinase antibodies and anti-human stromelysin and anti-human PUMP and mixtures thereof.
5
6. The diagnostic agent of Claim 1, wherein said inhibitors of matrix metalloproteinases are selected from the group consisting of tissue inhibitor of metalloproteinase (TIMP-1), tissue inhibitor of metalloproteinase (TIMP-2)
5 alpha-2 macroglobulin and mixtures thereof.

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7. The diagnostic agent of Claim 1, wherein said second substance is a polyclonal antibody select from the group consisting of rabbit anti-TIMP-1, rabbit anti-TIMP-2 and anti-alpha-2 macroglobulin.

8. The diagnostic agent of Claim 1, wherein said second substance is a monoclonal antibody to one of TIMP-1, TIMP-2 and mixtures thereof.

9. The diagnostic agent of Claim 5, wherein said monoclonal antibody is selected from the group consisting of antibodies to fragments of gelatinase A or gelatinase B.

10. The diagnostic agent of Claim 1, wherein said first substance is present in an amount of from about 0.01ng/ml to about 2000 ng/ml by weight of said biological sample and said second substance is present in an amount of from about 0.01
5 ng/ml to about 2000 ng/ml by weight of said biological sample.

11. The diagnostic agent of Claim 10, wherein said first substance is present in an amount of from about 0.01 ng/ml to about 2000 ng/ml by weight of said biological sample and said second substance is present in an amount of from about 0.01
5 ng/ml to about 2000 ng/ml by weight of said biological sample.

12. A method for detecting the presence of metastatic activity in a biologic sample, comprising:

contacting said biological sample with a diagnostic agent capable of indicating the presence of matrix
5 metalloproteinases (MMP's) and tissue inhibitors of MMPs.

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13. The method of Claim 12, wherein said matrix metalloproteinases are selected from the group consisting of 72 kDa type IV collagenase/gelatinase A (MMP-2), 92 kDa type IV collagenase/gelatinase B (MMP-9) and stromelysin (MMP-3) and putative metalloproteinase (PUMP).

14. The method of Claim 12, wherein said first responsive substance is capable of reacting with breakdown products of matrix metalloproteinases.

15. The method of Claim 12, wherein said first responsive substance is selected from the group consisting of monoclonal antibodies, polyclonal antibodies and mixtures thereof.

16. The method of Claim 15, wherein said first responsive substance is selected from the group consisting of murine monoclonal anti-human 72 kDa type IV gelatinase antibodies, 92 kDa type IV gelatinase antibodies and anti-human stromelysin antibodies and anti-human PUMP antibodies.

17. The method of Claim 12, wherein said inhibitors of matrix metalloproteinases are selected from the group consisting of tissue inhibitor of metalloproteinase (TIMP-1), tissue inhibitor of metalloproteinase (TIMP-2) and alpha 2 macroglobulin.

18. The method of Claim 17, wherein said second substance is a polyclonal antibody selected from the group consisting of rabbit anti-TIMP-1, rabbit anti-TIMP-2 and anti-alpha-2 macroglobulin.

19. The method of Claim 12, wherein said second substance is a monoclonal antibody to one of TIMP-1, TIMP-2 and mixtures thereof.

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20. The method of Claim 16, wherein said monoclonal antibody is selected from the group consisting of antibodies to fragments of gelatinase A or gelatinase B.

21. A kit for detecting the presence of metastatic disease in a biologic sample, comprising:

a) a first means for detecting the presence of matrix metalloproteinases;

5 b) a second means for detecting the presence of endogenous inhibitors of matrix metalloproteinases thereof; and

10 c) means for measuring the amount of matrix metalloproteinases detected by said first and said second means.

22. The kit of Claim 21, wherein said first means is an antibody against said matrix metalloproteinases and wherein said second means is an antibody against said tissue inhibitors of matrix metalloproteinases.

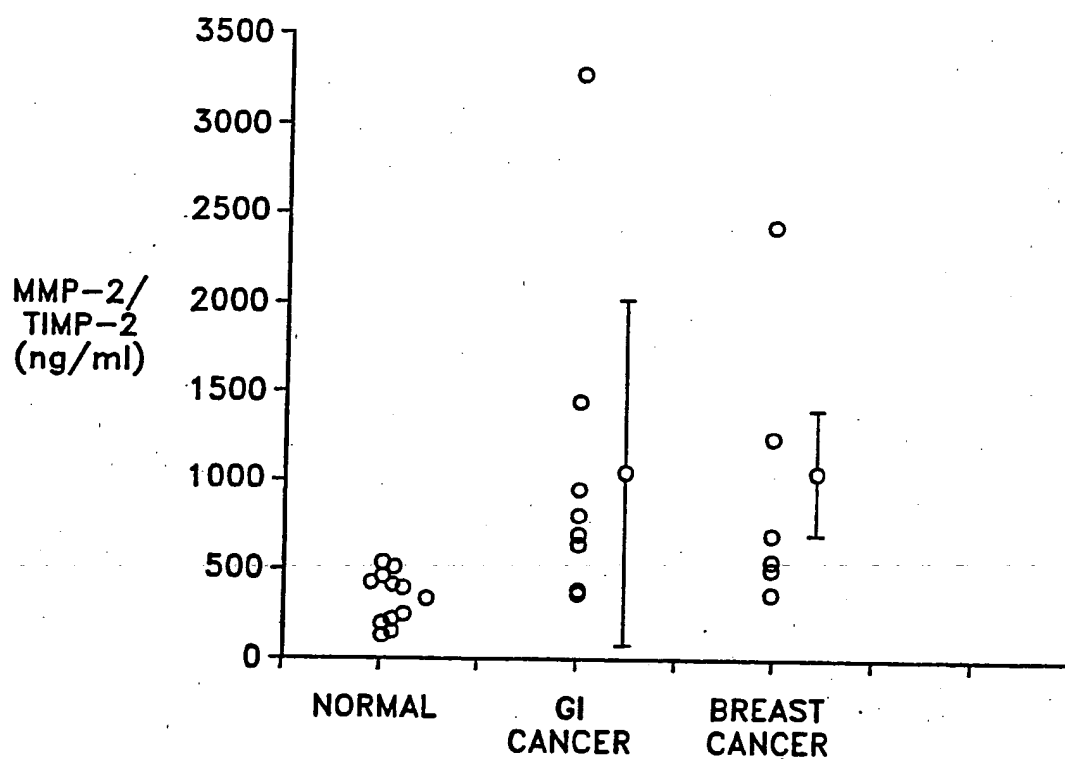
23. The kit of Claim 21, wherein said means is a monoclonal antibody and said second means is a monoclonal or polyclonal antibody.

24. The kit of Claim 21, wherein said means for measuring is an immunoassay.

25. The kit of Claim 24, wherein said means for measuring is an enzyme linked immunosorbent assay.

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FIG-1



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02967

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : G01N 33/574; C07K 15/28; C12Q 1/37
US CL : 435/7.23, 7.92, 7.94, 23; 530/387.1, 388.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.23, 7.92, 7.94, 23, 810; 436/518; 530/387.1, 388.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---------------|--|---|
| <u>X</u> Y | MATRIX, Volume 10, issued 1990, S. Cooksley et al., "Immunoassays for the Detection of Human Collagenase, Stromelysin, Tissue Inhibitor of Metalloproteinases (TIMP) and Enzyme-Inhibitor Complexes," pages 285-291, especially page 285, column 1, first paragraph; page 286, column 1 and column 2, first paragraph. | <u>1, 4, 12, 15</u> 2, 3, 5-11, 13, 14, 16-25 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | |
|---|--|
| * Special categories of cited documents: | * T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| * A document defining the general state of the art which is not considered to be part of particular relevance | * X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| * E earlier document published on or after the international filing date | * Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| * L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | * & document member of the same patent family |
| * O document referring to an oral disclosure, use, exhibition or other means | |
| * P document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

23 JUNE 1993

Date of mailing of the international search report

30 JUN 1993

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02967

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|---------------------------|
| Y | J. Biol. Chem, Volume 264, number 29, issued 1989, W.G. Stetler-Stevenson et al., "Tissue Inhibitor of Metalloproteinase (TIMP-2)," especially page 17347, column 1, first paragraph and column 2; page 17377, column 2. | 2, 3, 5-11, 13, 14, 16-25 |

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/02967

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Dialog: Biosis, CAB Abstracts, Medline, Embase, Cancerlit, Derwent

search terms: metastas?, plasma, metalloproteinase, MMP, collagenase, gelatinase, stromelysin, PUMP, macroglobulin, tissue(w)inhibitor, metallophthalcin

Form PCT/ISA/210 (extra sheet)(July 1992)*

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